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Original Article

Mechanisms underlying the wound healing potential of propolis based on its *in vitro* antioxidant activity

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ABSTRACT

Background: Propolis is a resinous substance collected by honeybees, *Apis mellifera*, from various plant sources. Having various pharmacological and biological activities, it has been used in folk medicine and complementary therapies since ancient times.
Purpose: To evaluate the effects and underlying mechanism of the protective effects of the ethanol extract of Chinese propolis (EECP) on L929 cells injured by hydrogen peroxide (H_2O_2).
Study design: The wound healing activities of EECP in L929 cells with H_2O_2-induced damage were investigated.
Methods: The main components of EECP were analyzed by RP-HPLC, and the free radical scavenging capacity and reducing power were also measured. The effects of EECP on the expression of antioxidant-related genes in fibroblast L929 cells were determined using qRT-PCR and western blotting.
Results: EECP had significant protective effects against cell death induced by H_2O_2 and significantly inhibited the decline of collagen mRNA expression caused by H_2O_2 in L929 cells.
Conclusion: EECP induced the expression of antioxidant-related genes, such as HO-1, GCLM, and GCLC, which has great implications for the potential of propolis to alleviate oxidative stress in wound tissues. The protective effects of propolis have great implications for using propolis as a wound healing regent.

Introduction

Skin is the largest organ of the body and acts as a wall to protect from attacks from various external factors, such as ultraviolet radiation (UVR), chemical toxics, microorganisms, and so on (Proksch et al., 2008). Therefore, the skin itself is directly impacted by toxic injuries, which cause adverse effects, such as erythema, edema, wrinkling, photoaging, inflammation and wound healing impairment (Nachbar and Korting, 1995; Parihar et al., 2008). Numerous reports have indicated that reactive oxygen species (ROS) appear to play a vital role in the pathogenesis of cutaneous wound healing (Bryan et al., 2012; Huo et al., 2009). ROS are generally produced during normal skin tissue metabolism and are kept at very low level by the strong oxidation–reduction system; therefore, they have few damaging effects (Kohen, 1999). However, defense mechanisms, though highly efficient (Kohen and Gati, 2000), have their limitations and may be overwhelmed when exposed to excessive levels of oxidative species. Uncontrolled release and inefficient removal of ROS may cause biomolecular oxidative damage and induce aberrant signal transduction, which contributes to an array of physiological manifestations in cells and tissues (Ichihashi et al., 2003). Thus, antioxidant therapy is believed to have significant benefits for improving oxidative stress-related cutaneous wound healing (Kant et al., 2014; Pinnell, 2003).

Propolis, a resinous substance, is collected by *Apis mellifera* from plant shoots or trunk lesions. The resin is mixed with mandibular gland secretions and beeswax. It has extensive plant sources and complex chemical compositions, including flavonoids, phenolic acids, terpenes, sugars, hydrocarbons and mineral elements (Bankova et al., 2000), which enable it to perform a wide range of pharmacological activities. Among these biological activities, the antioxidant activity of propolis has been widely studied. The chemical structure of the constituent polyphenols enable propolis to effectively eliminate free radicals.

Abbreviations: EECP, Ethanol extract of Chinese propolis; HPLC, High performance liquid chromatography; H_2O_2, Hydrogen peroxide; HO-1, Heme oxygenase-1; GCLC, Glutathione-cysteine ligase catalytic subunits; GCLM, Glutathione-cysteine ligase modify subunits; ROS, Reactive oxygen species; COL1A2, Alpha2 chain of type I collagen; COL3A1, Alpha1 chain of type III collagen

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Propolis promotes skin wound healing by stimulating epithelial regeneration (Pessolato et al., 2011), modulating extracellular matrix (collagen etc.) deposition (Olczyk et al., 2012; Olczyk et al., 2013b), and facilitating the formation of granulation tissue (Han et al., 2005). Hence, the antioxidant activity of propolis may contribute to its protective effects in cutaneous diseases. Burn wounds treated with propolis were found to have lower concentrations of free radicals (Olczyk et al., 2013a). In addition, it was reported that propolis could alleviate cell damage in fibroblast cells by suppressing intracellular ROS production induced by excessive light (Murase et al., 2013). Propolis has been used as a folk medicine to treat burns, ulcers and wounds for a long time (Kuropatnicki et al., 2013). It has been recorded that propolis was used to treat wounds during the Boer war in the early 1900 s (Ghisalberti, 1979).

However, few studies have been carried out to explore the mechanisms underlying the wound healing potential of propolis. In consideration of the crucial role that oxidative stress plays in skin damage, an *in vitro* study was conducted to gather additional evidence to support the clinical application of propolis in wound healing.

Composition variations exist between propolis from different plant origins, allowing it to exhibit different pharmacological activities. Our previous study demonstrated that poplar type propolis possesses strong anti-oxidant activity due to the abundant active polyphenols that it contained (Wang et al., 2013; Zhang et al., 2016). Thus, in this study, we investigated the antioxidant activity of Chinese propolis (poplar type) and its protective effects on hydrogen peroxide-induced changes (cell viability and collagen genes expression) in mouse L929 fibroblasts, and further examined the molecular mechanisms behind it. To our knowledge, this is the first report using Chinese propolis to evaluate the *in-vitro* protective effects of propolis against oxidative injury in fibroblasts.

Materials and methods

Materials

An alkaline phosphatase-conjugated secondary antibody (anti-rabbit IgG), DPPH, ABTS, α-tocopherol (Vitamin E), Trolox and the standards used in the HPLC analysis were purchased from Sigma (St. Louis, MO). Primary rabbit antibodies against HO-1 (lot #: YJ071709CS, monoclonal), GCLM (lot #: 5529–1, monoclonal), β-tubulin (lot #: YH082302D, monoclonal), and other analytical grade chemicals were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China).

Sample collection and extraction

Poplar type propolis (*Populus* spp.) was collected from *Apis mellifera* colonies in Shandong province of China during the summer of 2010. A voucher specimen of the samples (no. 130520) was deposited at the College of Animal Sciences, Zhejiang University. Samples were stored at −20 °C until used. Briefly, propolis samples were weighed and broken into powder form using a grinder, extracted with 95% (v/v) ethanol three times, and sonicated at 40 so for 3 h. Subsequently, the supernatants were filtered using Whatman No. 4 filter papers. The residues were extracted with 95% ethanol, the supernatants were collected and evaporated in a rotary evaporator under reduced pressure at 50 °C. After drying, the residues were collected and weighed. All samples were stored at −20 °C and redissolved in ethanol and filtered with a 0.22-μm syringe filter before use. During the cell experiments, the final concentration of ethanol in the medium did not exceed 0.1% (v/v).

HPLC analysis of propolis

To separate and determine the 24 main constituents of EECP, including flavonoids and phenolic acids, HPLC was conducted according to previously described methods (Cui-ping et al., 2014; Kumazawa et al., 2003). A Sepax HP-C18 column (150 mm × 4.6 mm, 5 μm; Sepax Technologies, Inc., Newark, DE) was used. The mobile phase was composed of 1.0% aqueous acetic acid (v/v) (A) and methanol (B) in gradient mode at 33 °C as follows: 15–40% (B) at 0–30 min, 40–55% (B) at 30–65 min, 55–62% (B) at 65–70 min, and 62–100% (B) at 70–85 min at a flow rate of 1.0 ml/min. The injection volume was 5 μl, and the results were detected at 280 nm.

Determination of the free radical scavenging activities and reducing power

DPPH radical scavenging activity (DPPH)

The hydrogen donating activity was measured by direct hydrogen donation to the DPPH radical as described in a previous report with minor modifications (Yang et al., 2011). The reaction solution consisted of 100 μL of sample and 100 μl of DPPH solution; 100 μl of the mixture per well was incubated in a 96-well plate at room temperature for 30 min in the dark. All experiments were performed in triplicate. The absorbance was detected at 517 nm, and the results are expressed as IC50 values (μg/ml).

ABTS cation radical scavenging activity (ABTS)

The ABTS radical-scavenging activity assay was performed according to the modified method (Yang et al., 2011). The ABTS working reagent was diluted with methanol to reach an absorbance of 0.7 at 734 nm. 50 μl of sample and 100 μl of the ABTS working solution were aliquoted into the 96-well plate away from light for 16 h before use, and the absorbance was measured at 734 nm after being incubated for 10 min in the dark. All experiments were performed in triplicate. The scavenging ability results were expressed as IC50 values (μg/ml).

Measurement of the reducing power (RP)

The power of reducing ferric ions was measured by a modified method (Moreira et al., 2008). 125 ml of the propolis sample was mixed with 312.5 μL of phosphate buffer (0.2 M, pH 6.6) and 312.5 ml of 1% potassium ferrocyanate. The mixture was preheated at 50 °C for 20 min, followed by the addition of 312.5 μl of 10% trichloroacetic acid, and then centrifuged at 2000 rpm for 10 min. The reaction solution consisted of 1 ml supernatant, 312.5 μl of distilled water and 62.5 μl of 0.1% ferric chloride. Four-hundred microliters of the reaction solution was pipetted into a 96-well plate (200 μL/well). The absorbance was detected at 700 nm, and trolox was used as the reference sample. The results were expressed as the Trolox equivalent (mmol) per gram of propolis.

Cell culture and cell viability assay

Fibroblasts L929 cells were incubated in DMEM (Keyi Biotechnology Company, Hangzhou, China) containing 10% fetal bovine serum (Gibco, Grand Island, NY) at 37 °C and 5% CO_2 in a humidified incubator. The toxicity of EECP and H_2O_2 was determined by using a CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 550, CA).

ROS levels in L929 cells

Fibroblast L929 cells were cultured with the indicated concentrations of propolis for 3 h, followed by stimulation with 600 μM H_2O_2 for 12 h. Next, the cells were washed twice with PBS to remove extracellular reactive oxygen species (ROS) and incubated with a new culture coupled with 200 μM DCHF-DA for 30 min. The cells were removed from the culture medium and washed with PBS, collected using trypsin and centrifuged at 2500 rpm for 5 min to remove extracellular compounds. Next, the ROS levels were determined by a BD FACS Calibur (Franklin, NJ).

Table 1
Sequences of the primers for qRT-PCR.

Genes	Sense primers	Antisense primers
HO-1	5′-ACATTGAGCTGTTTGAGGAG-3′	5′-TACATGGCATAAATTCCCACTG-3′
GCLM	5′-CTGACATTGAAGCCCAGGAT-3′	5′-GTTCCAGACAACAGCAGGTC-3′
GCLC	5′-GATGATGCCAACGAGTCTGA-3′	5′-GACAGCGGAATGAGGAAGTC-3′
GAPDH	5′-GAGAAACCTGCCAAGTATGATGAC-3′	5′-TAGCCGTATTCATTGTCATACCAG-3′

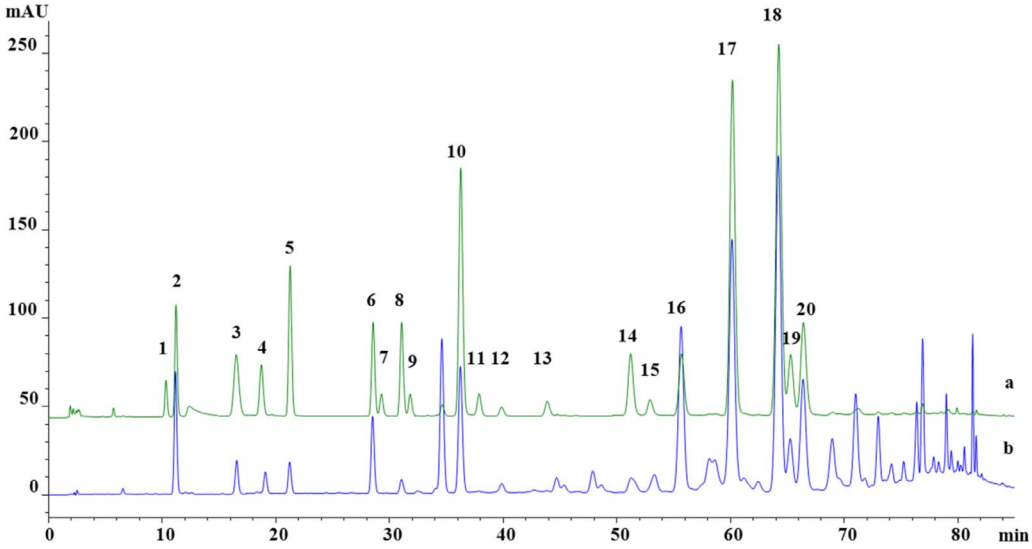

Fig. 1. The HPLC chromatograms of the standard solution (a) and EECP (b) at a wavelength of 280 nm. Peaks represent: 1. Vanillic acid; 2. Caffeic acid; 3. p-Coumaric acid; 4. Ferulic acid; 5. Isoferulic acid; 6. 3,4-Dimethoxycinnamic acid; 7. Rutin; 8. Cinnamic acid; 9. Myricetin. 10. Pinobanksin; 11.Naringenin; 12. Quercetin; 13. Luteolin; 14. Kaempferol. 15. Apigenin; 16. Pinocembrin; 17. 3-O-acetylpinobanksin; 18. Chrysin; 19. Caffeic acid phenylethyl ester; 20. Galangin.

Quantitative real-time polymerase chain reaction analysis

After treating L929 cells with certain concentrations of EECP, the culture medium was removed and total RNA was isolated using a RNA pure Total RNA kit (Aidlab, Beijing, China) according to the manufacturer's instructions. cDNA (1 μg of RNA) was synthesized using a primeScriptTM RT Reagent kit (TaKaRa, Dalian, China) and diluted (1:25) to conduct quantitative real-time polymerase chain reaction (qRT-PCR) analysis, at a final volume of 7 μl with the Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using SYBR Premix Ex TagTM (TaKaRa). The reaction conditions were as follows: 95 °C for 30 s, 95 °C for 5 s and 60 °C for 30 s, followed by the melting curve analysis at 95E °C for 15 s, 50 °C for 15 s and 95E °C for 15 s. GAPDH, which is a housekeeping gene, was used as a control to normalize the expression of the target genes. The specificity was confirmed by dissociation curve analysis and gel electrophoresis. All of the oligonucleotide primers were designed and synthesized by Sangon Biotech. The sequences of the primers are shown in Table 1.

Preparation of protein and western blotting

L929 cells were treated with EECP (10 μg/ml) for the indicated lengths of time and the expression of the antioxidant-related genes (HO-1, GCLC, GCLM) were determined. On the other hand, L929 cells were pretreated with the assigned concentrations of EECP for 3 h before stimulating with H_2O_2 (0.8 mM) for the indicated lengths of time. At the time of harvest, cells were put on ice and washed twice with cold PBS immediately. The cytoplasmic proteins were lysed with NP40 mixed with protease inhibitors and phosphatase inhibitors, and the cell lysate was collected using cell scrapers (Corning, New York), vortexed and put on ice for 10 min. Next, the lysate was centrifuged at 16,000 rpm for 10 min at 4 °C. Subsequently, the supernatants were collected and added to a certain volume of Laemmli's sample buffer and then boiled at 95 °C for 10 min. The concentration of protein was determined using a BCA protein assay kit (Weiao Biotechnology, Shanghai, China). The proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); then, the gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The PVDF membrane was incubated with 5% non-fat milk for 1 h at room temperature to block the nonspecific binding sites. The blots were then incubated with primary antibodies. After washing three times with Tris-buffered saline Tween 20, the membranes were incubated with an alkaline phosphatase-conjugated secondary antibody for another 1 h The membranes were washed three times and developed using the method described by Chen et al. (Chen et al., 2016).

Statistical analysis

The results are expressed as the mean ± SD, and each mean is representative of three independent experiments. Statistical analysis was conducted using Student's *t*-test or one-way ANOVA with the Student–Newman–Keules method performed using the SPSS software (Chicago, IL) to determine significant differences. Values of $P < 0.05$ were considered statistically significant.

Results

Determination of the composition of EECP by HPLC analysis

Propolis is rich in polyphenols which contributes to its multiple biological activities. Based on a previous study, we measured the presence of 20 common compounds in EECP by HPLC analysis, as shown in Fig. 1 and Table 2, and the results indicated that EECP was rich in flavonoids, among which pinocembrin, 3-O-acetylpinobanksin, and chrysin showed the highest levels. In addition, vanillic acid, rutin and myricetin were not detected in EECP.

Free radical scavenging activities and reducing power of EECP

DPPH and ABTS radical scavenging assays and the ferric reducing

Table 2
Contents of the 20 compounds presented in EECP.[a]

Compounds	Retention Time (min)	mg/g of extract
Vanillic acid	10.41	–
Caffeic acid	11.18	7.42 ± 2.34
p-Coumaric acid	16.53	1.71 ± 0.49
Ferulic acid	19.02	1.49 ± 0.21
Isoferulic acid	21.17	2.61 ± 0.09
3,4-Dimethoxycinnamic acid	28.50	7.93 ± 1.19
Rutin	29.19	–
Cinnamic acid	31.05	0.52 ± 0.09
Myricetin	32.02	–
Pinobanksin	36.22	14.74 ± 1.89
Naringenin	38.24	0.59 ± 0.12
Quercetin	40.17	3.14 ± 0.38
Luteolin	44.64	2.59 ± 0.68
Kaempferol	47.82	2.14 ± 0.32
Apigenin	51.19	4.10 ± 0.98
Pinocembrin	55.61	26.73 ± 6.71
3-O-acetylpinobanksin	60.08	53.53 ± 5.29
Chrysin	64.18	37.81 ± 3.13
CAPE	65.22	12.30 ± 5.71
Galangin	66.36	12.03 ± 2.64

[a] Reported values are the means ± SD (n = 3); CAPE, caffeic acid phenylethyl ester; "-", not detected.

Table 3
Radical scavenging capacity and reducing power of EECP.[a]

Sample (μg/ml)	DPPH (IC_{50})	ABTS (IC_{50})	RP (mmol Trolox/g)
EECP	47.71* ± 1.34	110.28** ± 0.63	1.73* ± 0.09
α-Tocopherol	38.23 ± 0.39	78.89 ± 0.67	1.57 ± 0.04

[a] DPPH and the ABTS free radical scavenging capacity are expressed by IC_{50} values (μg/ml); RP, reducing power, was expressed as millimoles Trolox equivalents (TE) per gram of sample; Data are expressed as the mean ± SD (n = 3); $^{*}P < 0.05$; $^{**}P < 0.01$ versus the α-Tocopherol group.

power test have been commonly used to evaluate the antioxidant capacity of propolis extracts and other natural plant constituents (Giampieri et al., 2014; Moreira et al., 2008). In this study, we evaluated the radical scavenging abilities and reducing power of EECP, with α-Tocopherol as the reference group. As shown in Table 3, we observed that EECP showed highly significant radical scavenging effects, but relatively weaker effects compared to those of α-Tocopherol. However, EECP had a better ferric reducing power than that of α-Tocopherol.

Effect of EECP on cell proliferation and vitality of L929 cells

Cell proliferation and the vitality of skin cells are crucial during the wound healing process. As shown in Fig. 2, our results demonstrated that EECP had no growth stimulation effects on L929 cells. At a high concentration of 20 μg/ml, EECP had cytotoxic effects on L929 cells, with a cell survival rate of 83% ($P < 0.05$). Similar results were observed with other types of propolis ethanol extracts, some single ingredients in propolis, and other types of fibroblast cells (data not shown). In our study, EECP showed no cytotoxicity when its final concentration was lower than 10 μg/ml. Therefore, all further experiments were conducted at this safe concentration of EECP.

The cytotoxicity of H_2O_2 and the protection of EECP on H_2O_2-stimulated L929 cells

The previous results indicated that propolis probably improves skin wound healing in ways other than exerting positive effects on cell growth, such as protecting cells from harmful stimulations. Considering the negative effects of oxidative stress on wound healing, we suspected that protecting skin cells from the damage caused by ROS may at least

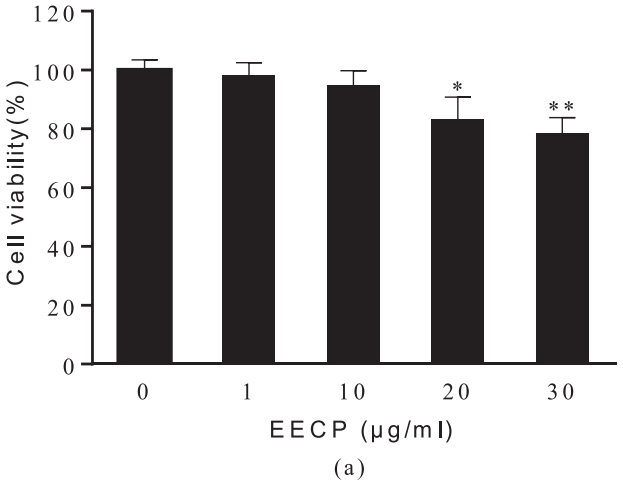

Fig. 2. Effect of EECP on the cell proliferation and vitality of fibroblasts L929 cells. Cells were pretreated with/without the indicated concentrations of EECP (0–30 μg/ml) for 24 h The results are expressed as the percentages of surviving cells over control cells (untreated group) detected using the CCK8 assay. Each result is expressed as the mean ± SD (n = 3); $^{*}P < 0.05$; $^{**}P < 0.01$ versus the untreated group.

partly explain the mechanisms underlying the wound healing potential of propolis. To confirm our suspicion, we investigated the effects of EECP on H_2O_2-induced cytotoxicity in L929 cells.

We first examined the effects of H_2O_2 at different concentrations on the viability of L929 cells, and the results, as shown in Fig. 3(a), indicated that H_2O_2 has a dose-dependent inhibition effect on cell viability, with stimulation concentrations ranging from 0.3–0.9 mM. When the concentration is lower than 0.6 mM, H_2O_2 may decrease cell vitality by primarily inhibiting cell proliferation, as few numbers of dead cells were observed under the microscope at lower concentrations. When the H_2O_2 concentration was 0.7 mM, the survival rate was approximately 18.7% ($P < 0.01$) and characteristic apoptosis and necrosis features, including shrinkage, going round and falling off, were observed in cells under the microscope. Only approximately 8% ($P < 0.01$) of cells survived after 24 h of stimulation with 0.8 mM H_2O_2.

Next, we examined the viability of L929 cells stimulated by 0.8 mM H_2O_2 for 24 h with or without 3 h of pretreatment with EECP. As demonstrated in Fig. 3 (b-c), the decline of the cell survival rate caused by high levels of H_2O_2 could be prevented when cells were pretreated with EECP, which suggested the strong protective effects of propolis extract on ROS-induced cell death. The protective effects improved with increasing concentrations of EECP. The survival rate was increased to 90.03% ($P < 0.01$) when cells were pre-incubated with 10 μg/ml EECP.

Protective effects of EECP against H_2O_2-induced changes in COL1A2 and COL3A1 mRNA expression

Collagen is an important component of connective tissue, providing tensility and elasticity to the skin. Exposure to UVR causes collagen reduction in the dermis, impairs human skin and causes premature skin aging (photoaging), which leads to a wrinkled appearance(Chen et al., 2015). Moreover, the synthesis, deposition, and remodeling of collagen play pivotal roles in cutaneous wound healing. However, excessive or insufficient production of collagen during some pathological processes cause fibrosis diseases or repair disorders. It has been demonstrated that collagen production plays important roles during the development of wound repair(Chattopadhyay and Raines, 2014).Therefore, we further explored the effects of EECP on H_2O_2-induced changes in COL1A2 (the α2 chain of collagen type I) and COL3A1 (the α1 chain of collagen type III) mRNA expression. The results are shown in Fig. 4. When L929 cells were stimulated using 0.6 mM H_2O_2 for 12 h, the relative COL1A2

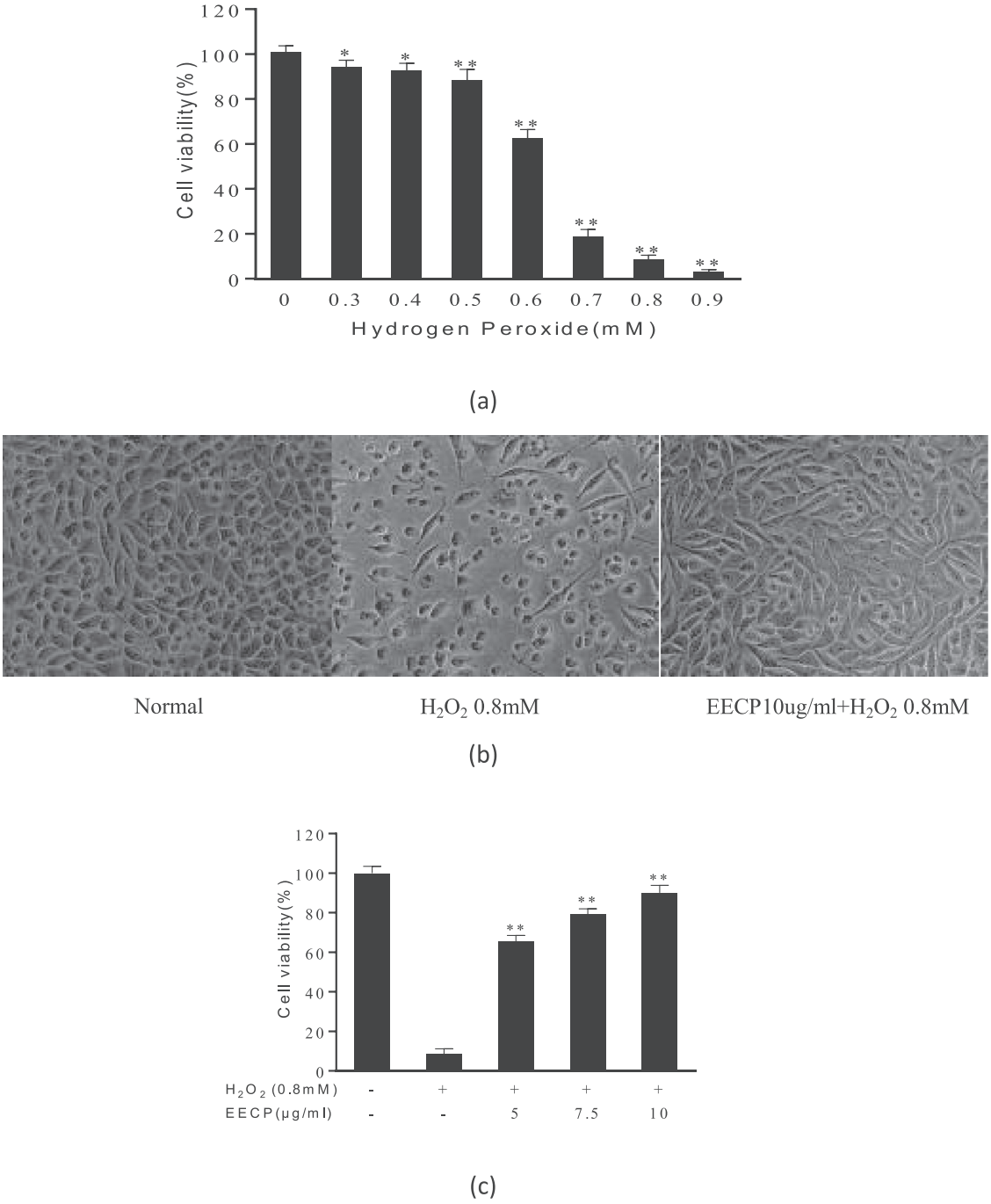

Fig. 3. The cytotoxicity of H_2O_2 and protection of EECP on H_2O_2-stimulated L929 cells. (a) Cells were pretreated with/without the indicated concentrations of H_2O_2 (0.3 mM-0.9 mM) for 24 h (b) Representative images indicating the protective effects of EECP on H_2O_2-treated L929 cells. Images were captured at 10 × magnification using a phase-contrast microscope (Nikon EclipseTS100, Japan). (c) Cells were pretreated with/without the indicated concentrations of EECP for 3 h and then stimulated with 0.8 mM H_2O_2 for 24 h The results are expressed as the percentage of surviving cells over control cells (untreated group) detected by the CCK8 assay. Each result was expressed as the mean ± SD ($n = 3$); $^{**}P < 0.01$ versus the H_2O_2 group.

mRNA expression was equivalent to 0.51 times ($P < 0.05$) that of the normal group. When L929 cells were pretreated with EECP for 3 h, the decline of COL1A2 mRNA expression induced by H_2O_2 was prevented in a dose-dependent manner. The relative COL1A2 mRNA expression increased to 0.75 times ($P < 0.05$) that E the normal group when cells were pre-incubated with 10 μg/ml EECP. Similar dose-dependent protective effects were observed for COL3A1 mRNA expression.

Inhibitory effects of EECP on the production of ROS in L929 cells

H_2O_2 may generate stronger ROS (•OH, etc.) by the Fenton Reaction, causing severe oxidative stress injury to cells (Farber, 1994; Imlay, 2003). Previous studies demonstrated that H_2O_2 at high concentrations were able to reduce collagen expression and cell viability, and EECP prevented those changes. We suspected that EECP probably helped eliminate excessive ROS produced in cells, consequently reducing oxidative stress injuries caused by H_2O_2. To confirm our suspicion, we detected cellular ROS levels using an ROS-sensitive fluorescent probe called DCFH-DA.

The results are shown in Fig. 5. The cellular ROS levels were remarkably increased after 12 h of stimulation with 0.6 mM H_2O_2, and EECP was able to inhibit these changes effectively in a dose-dependent

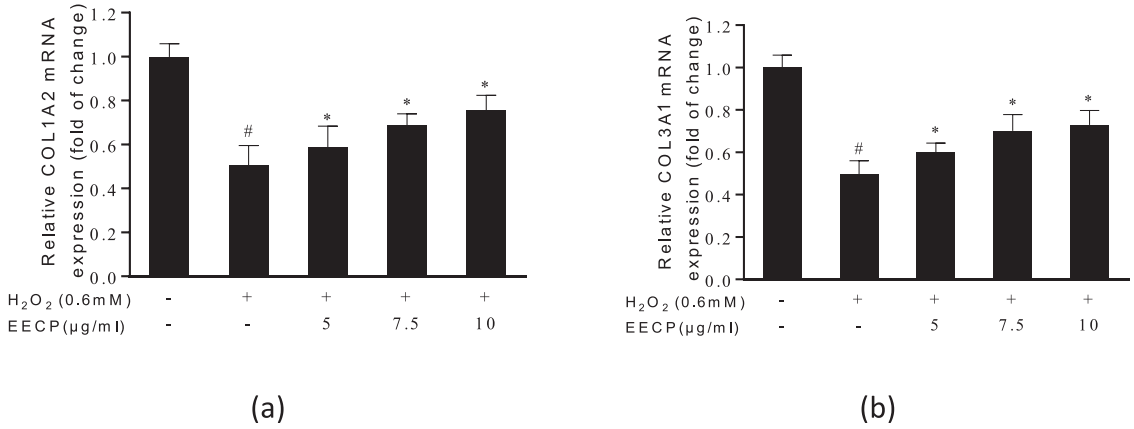

Fig. 4. Effect of EECP on H_2O_2-induced changes in COL1A2 and COL3A1 mRNA expression. L929 cells were pretreated with or without the indicated concentrations of EECP for 3 h and were then stimulated with 0.6 mM H_2O_2 for 12 h The relative mRNA expression of COL1A2 (a) and COL3A1 (b) were determined using qRT-PCR. COL1A2 represents the α2 chain of type I collagen; COL3A1 represents the α1 chain of type III collagen; each result was expressed as the mean ± SD ($n = 3$). $^{\#}P < 0.05$ versus the untreated group, $^{*}P < 0.05$ versus the H_2O_2 group.

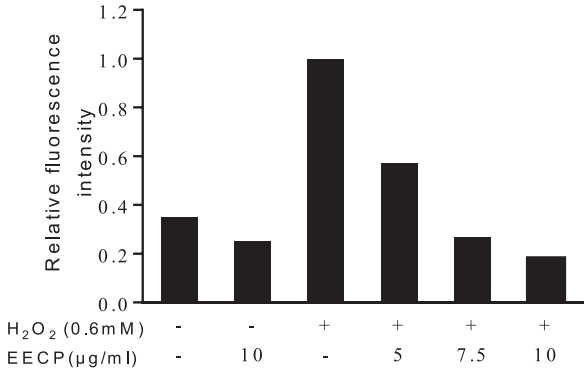

Fig. 5. Effect of EECP on H_2O_2-induced ROS level changes in fibroblasts L929 cells. L929 cells were pretreated with or without the indicated concentrations of EECP for 3 h and then stimulated with 0.6 mM H_2O_2 for 12 h Cells treated with only EECP or not treated were used as controls. The intracellular ROS levels were determined using a DCFH-DA fluorescent probe and flow cytometry. The representative results were chosen from three independent experiments with similar results and expressed as the relative fluorescent intensity.

manner, thereby confirming our suspicion.

Effects of EECP on the mRNA expression of antioxidant genes (HO-1, GCLM, and GCLC)

Cutaneous injury initiates a series of events, including inflammation, new tissue formation, and matrix remodeling. In the early stages of inflammation, abundant neutrophils and macrophages migrate to the wounded tissue. Once activated, they produce large amounts of ROS to defend against invading bacteria. In addition to their beneficial role in microbial killing, increased levels of ROS can also inhibit cell migration and proliferation and can cause severe tissue damage (Steiling et al., 1999). To protect wounded tissue from oxidative stress, production of antioxidant-related enzymes and small antioxidant molecules is promptly increased for the detoxification of excess ROS. Heme oxygenase-1 (HO-1) and glutathione (GSH) are two such important antioxidants (auf dem Keller et al., 2006). HO-1 is the rate-limiting enzyme in the degradation of heme into carbon, free iron, and biliverdin, which is subsequently reduced to bilirubin, a potent antioxidant agent (Schäfer and Werner, 2008). GSH is an endogenous antioxidant, and a glutamate-cysteine ligase (GCL), consisting of a GCL catalytic subunit (GCLC) and a GCL modulatory subunit (GCLM), is also the rate-limiting enzyme in GSH (Seelig et al., 1984). A previous report from our lab demonstrated that propolis ethanol extracts could promote the expression of the HO-1, GCLC and GCLM genes in mouse macrophage Raw 264.7 cells (Zhang et al., 2015). Therefore, we suspected that EECP probably protected L929 cells from oxidative injury by stimulating the expression of these antioxidant-related genes. In the following experiments, the relative mRNA expression of the HO-1, GCLC and GCLM genes was determined using qRT-PCR. As shown in Fig. 6 (a-c), EECP promoted the expression of these genes, and the expression levels of all of the genes reached a maximum at 6 h, before gradually returning to normal levels.

Effects of EECP on HO-1, GCLM protein expression in L929 cells

To further confirm the promotion effects of propolis on the expression of antioxidant-related genes, we studied the protein expression levels of HO-1 and GCLM after L929 cells were treated with EECP (10 μg/ml) for different lengths of time (0,3,6,9,12,24 h). The results, as shown in Fig. 7, demonstrate that EECP could distinctly promote the protein expression of HO-1and GCLM in L929 cells. The fundamental expression of HO-1 was very low, but its expression gradually increased and reached a maximum within 6–9 h of stimulation with EECP, followed by a decrease in expression, eventually returning to normal levels. The protein expression of GCLM also gradually increased and reached a maximum before it slowly recovered and returned to normal levels.

Discussion

In this study, the ethanol extract of Chinese propolis (EECP) was used to investigate the protective effects of propolis against oxidative injury in fibroblasts, with the aim of providing further evidence for the successful application of propolis application in treating oxidative stress-involved cutaneous disorders. The raw material used for EECP production was collected from Shandong province in North China, where plants of the poplar type widely exist. Based on a previous study, we analyzed 20 common compounds that present in EECP by HPLC, and the results indicated that EECP was rich in flavonoids, among which pinocembrin, 3-O-acetylpinobanksin, and chrysin showed the highest levels. Vanillic acid, rutin and myricetin were not detected in EECP, which was in perfect agreement with a previous study performed in our lab (Cui-ping et al., 2014).

Antioxidant activity is one of the basic activities of propolis, which has great implications for propolis exerting a wide range of pharmacological actions, such as liver protection (Bhadauria et al., 2008; Turkez et al., 2013), neuroprotection (Shimazawa et al., 2005) and wound healing (Olczyk et al., 2013a). In this study, we evaluated the

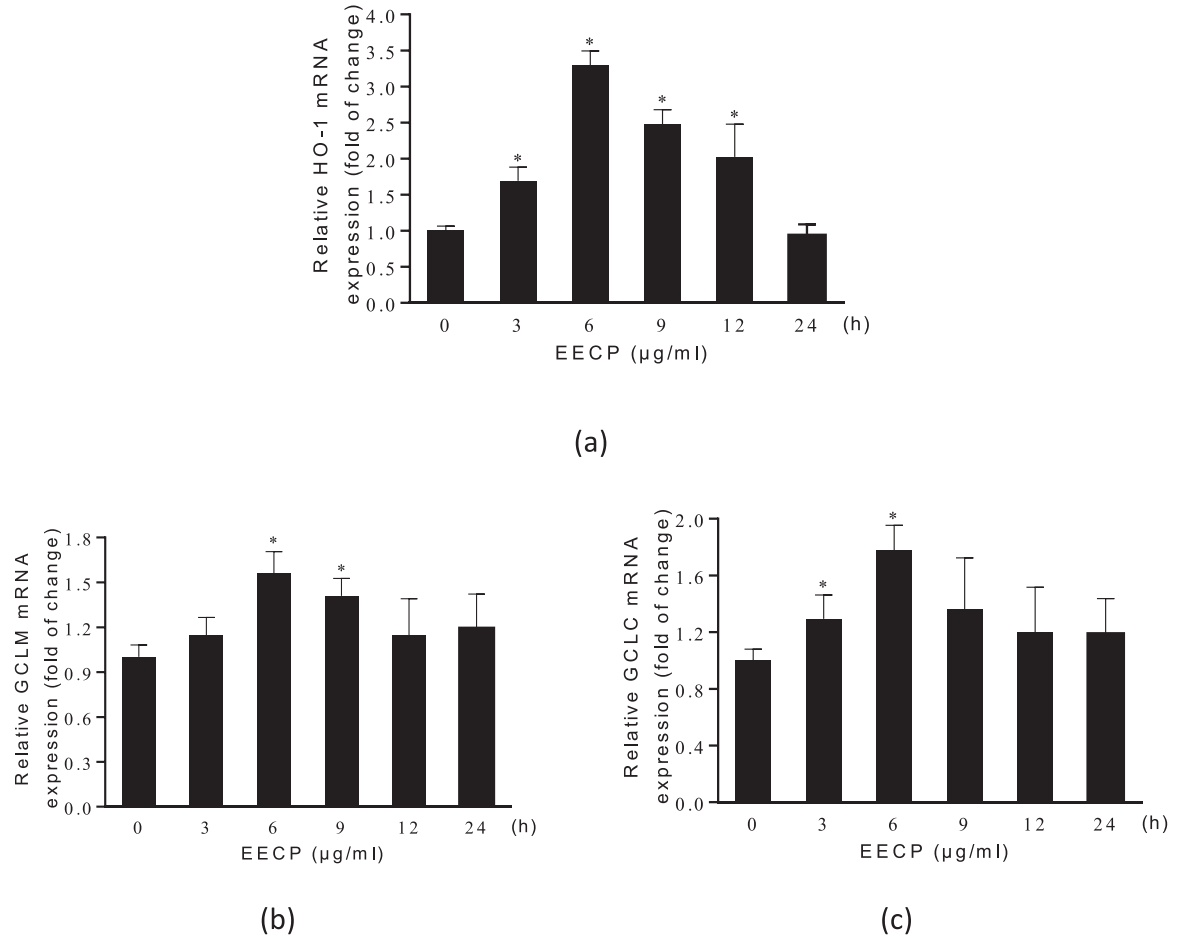

Fig. 6. Effects of EECP on mRNA expression of antioxidant-related genes (HO-1, GCLM, GCLC) in L929 cells. (a–c) Cells were treated with EECP (10 μg/ml) for indicated lengths of time, and the relative mRNA expression of antioxidant-related genes (HO-1, GCLM, GCLC) was determined using qRT-PCR. Each result is expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$ versus the untreated group.

radical scavenging abilities and reducing power of EECP. The inconformity between these antioxidant results may derive from the difference in assessment techniques used for those antioxidant assays. The excellent antioxidant activities of EECP can be explained by its rich phenolic components. Several flavonoids and phenolic acids in propolis, such as quercetin, kaempferol, galangin, caffeic acid, phenethyl caffeat, among others, have been reported to have significant antioxidant activities (Kumazawa et al., 2004).

It is known that ROS can exacerbate diseases in multiple ways, including causing cell injury, changing the expression of important genes, or directly inducing cell apoptosis and necrosis. To examine the mechanisms underlying the protective effects of propolis in skin disorders or diseases, such as UVR-induced skin photoaging, burn wounds, and so on, we examined the possible effects of EECP on H_2O_2-stimulated mouse skin fibroblasts L929. First, we detected the effects of EECP and H_2O_2 at different concentrations on the viability of L929 cells. Our results demonstrated that EECP may exert an inhibitory effects on the growth of L929 cells when the concentration value is beyond a certain level. It has been reported that flavonoids can act as signaling molecules to modulate some protein kinase and lipid kinase signaling pathways that usually play pivotal roles in the growth, differentiation and survival of cells (Williams et al., 2004). Therefore, at high doses, the flavonoid-rich ethanol extract of propolis may inhibit the growth of L929 cells by excessively suppressing or activating crucial signaling pathways. Additionally, our result highlights the importance of precise drug dose for treatment of diseases.

In our study, EECP showed no cytotoxicity when its final concentration was lower than 10 μg/ml. We then examined the effects of H_2O_2 at different concentrations on the viability of L929 cells, and the results indicated that H_2O_2 has a dose-dependent inhibitory effect on

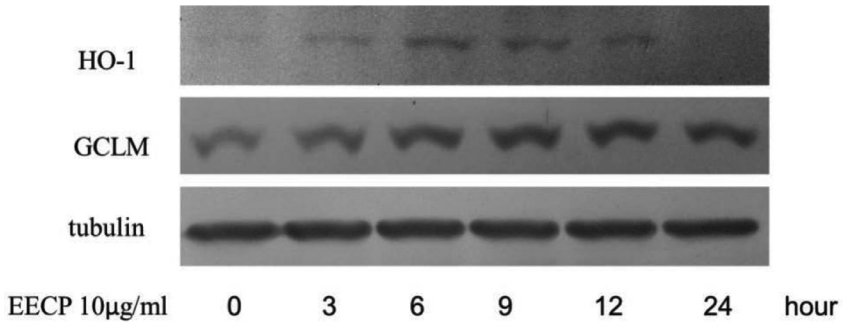

Fig. 7. Effects of EECP on the HO-1, GCLM protein expression levels in L929 cells. Cells were treated with EECP (10 μg/ml) for the indicated lengths of time, and the relative expression of antioxidant-related proteins (HO-1, GCLM) was determined using Western-blotting. Representative data from three experiments with similar results are shown.

cell viability, with stimulation concentrations ranging from 0.3–0.9 mM. However, as shown in Fig. 2, the decline of the cell survival rate caused by high levels of H_2O_2 could be prevented when cells were pretreated with EECP, which suggested the strong protective effects of propolis extract against ROS-induced cell death. In addition, collagen expression and secretion are the basic functions of skin fibroblasts, which contributes greatly to the maintenance of skin tissue strength, wound healing, and so on. Some reports have demonstrated that ROS may influence collagen expression in fibroblasts, resulting in the occurrence and development of cutaneous disorders (Emri et al., 2006; Galicka et al., 2014). Therefore, further study was undertaken to investigate the change in collagen biosynthesis caused by H_2O_2 in L929 cells pretreated with or without EECP. The qRT-PCR results demonstrated that mRNA expression of type I and type III collagen, which serve as structural and regulatory molecules in the skin, were inhibited by H_2O_2 alone and that inhibition was efficiently prevented when the cells were pretreated with EECP. In sum, EECP exerted strong protective effects on H_2O_2-induced injury of L929 cells by inhibiting cell death and a collagen expression decline. This may partly explain why burn wounds treated with propolis led to enhanced collagen depositions (Olczyk et al., 2013b), especially during the initial stages of wound healing, which are usually accompanied by severe oxidative stress (Parihar et al., 2008).

It has been reported that H_2O_2 injures cells as a result of the generation of highly potent oxidizing species, such as •OH, by the Fenton Reaction (Moriyama et al., 2015). In addition, ROS may not only act as toxic molecules but may also play multiple key roles as signaling molecules regulating a variety of biological processes (Veal et al., 2007). We hypothesized that EECP probably protected L929 cells from oxidative injury by modulating the intracellular ROS levels. To test our hypothesis, we determined the change in the intracellular ROS levels in H_2O_2-stimulated L929 cells with or without pretreatment of EECP. In the present study, we found that pretreating L929 cells with EECP potently suppressed excessive ROS accumulation induced by high levels of H_2O_2. This result is supported by our previous experiments that demonstrated that EECP has a significant radical scavenging capacity and reducing power. In other words, EECP probably prevented excessive ROS accumulation by its own antioxidant activity, which primarily depends on its constituent flavonoids and phenolic acids (Gong et al., 2010; Shirai et al., 2002).

However, an emerging view suggests that the classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation of the cytoprotection function of flavonoids, as many of them are also able to exert cellular effects by modulating protein kinase and lipid kinase signaling pathways (Williams et al., 2004). Our previous study demonstrated that EECP could protect mouse macrophages Raw 264.7 cells from oxidative injury by activating specific signaling pathways and therefore upregulating the expression of antioxidant-related genes (Zhang et al., 2015). In our study, we observed that EECP could stimulate the expression of antioxidant-related genes (HO-1, GCLC and GCLM) at the mRNA and protein level in skin fibroblasts. It should be noted that endogenous antioxidants and ROS detoxification enzymes, including GSH and HO-1, have great implications for skin morphogenesis and wound repair (Beyer et al., 2007). Some studies have demonstrated that inhibiting GSH synthesis reduces wound burst strength (Adamson et al., 1996). Another study indicated that maximal expression of HO-1 in the skin was observed on the 2nd and 3rd days after wounding in wild-type mice (Grochot-Przeczek et al., 2009). Lack of HO-1 could lead to the complete suppression of re-epithelialization and to the formation of extensive skin lesions, accompanied by impaired neovascularization. Interestingly, the water extract of propolis (WEP) and its major constituents were also found to increase the HO-1 expression levels after UV irradiation at earlier time points, which protected immortalized human skin fibroblast cells (NB1-RGB) against UVR-induced cell death (Murase et al., 2013; Saito et al., 2015). Therefore, the wound healing potential of propolis probably correlates with its capacity to promote the expression of antioxidant-related genes.

Conclusion

In the present study, we observed that oxidative stress induced by ROS had a strong negative impact on the vitality and collagen expression of skin fibroblasts, whereas propolis ethanol extracts efficiently reduced the excessive accumulation of ROS, protecting skin cells from oxidative injury. The protective effects of EECP involves two factors: free radical scavenging activities and signaling molecule-like characteristics, which promote the expression of some antioxidant-related genes. This partly explains why some studies reported that propolis could promote wound healing and protect skin from ultraviolet irradiation and also suggests the promising effects of propolis in treating other oxidative stress-involved skin diseases. However, further study is still required to elucidate the mechanisms underlying the wound healing potential of propolis to improve the development and clinical application of propolis.

Conflict of interest

The authors declare no conflict of interests regarding the publication of this paper.

Author contributions

X.C., Y.C. and K.W. conceived and designed the experiments; X.C., Y.C., and J.Z. performed the experiments; X.C. and M.Y. analyzed the data; K.W. and M.Y. contributed to the plant investigation/reagents/materials/analysis tools; X.C., Y.C. and F.H. wrote the paper.

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